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Salt-promoted adsorption of proteins onto amphiphilic agarose-based adsorbents
II. Effects of salt and salt concentration[1]

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Abstract

The effects of different types of salts and salt concentrations on the selectivity in the adsorption of serum proteins have been compared for the amphiphilic agarose-based adsorbents Phenyl-Sepharose®, Octyl-Sepharose®, butyl-agarose and mercaptopyridine-derivatized agarose. By use of multivariate analysis, the complex interrelationships for the different combined effects were evaluated. From these analyses conclusions about similarities and/or dissimilarities in the mechanisms involved in adsorption of proteins on respective adsorbent were made. © 1998 Elsevier Science B.V.

Keywords: Adsorption; Agarose-based adsorbents; Stationary phases, LC; Principal component analysis; Salt-promoted adsorption; Proteins

1. Introduction

Since the introduction of hydrophobic interaction chromatography (HIC) [1] and the classical hydrophobic adsorbents, few efforts have been concentrated on improving this technique for separation. Adsorbents used in HIC are exclusively derivatized with alkyl chains of different lengths [2] or aromatic groups [3]. Studies by Oscarsson et. al [4] on the commercially available hydrophobic adsorbents Octyl-Sepharose® and Phenyl-Sepharose® show that these adsorbents in some applications are too hydrophobic to be effective. Some adsorbed proteins are so firmly adsorbed that even more drastic conditions as desorption with ethylene glycol or sodium hydroxide are not efficient enough to desorb all protein bound to the gels. This is not surprising since hydrophobic surfaces are known to give rise to pronounced conformational changes of adsorbed proteins [5] with resulting difficulties to be desorbed. This paper concerns the possibilities of expanding the usefulness of HIC by introducing other types of ligands with complementary characteristics compared to existing HIC adsorbents and also by introducing other buffer systems than those commonly used.

Serum was used as sample in an attempt to investigate mercaptopyridine-derivatized agarose as an alternative ligand to the traditional adsorbents like

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[1]The raw data table can be obtained from the authors upon request. It will also be available via the J. Chromatogr. Editorial Office: e-mail chrom-eo@elsevier.nl

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phenyl-, octyl- or butyl-agarose. The use of serum makes the evaluation more complicated but at the same time more generalized conclusions can be made since the proteins studied represent a diversity of important properties for proteins interacting with surfaces. The evaluation was made by multivariate analysis [6] which made it possible to classify the investigated adsorbents according to the pattern of their respective interactive behavior – their selectivity.

2. Experimental

2.1. Chemicals

KCl, NaAc and K_2SO_4 were purchased from Fluka (Buchs, Switzerland), NaCl and NH_4Ac from Merck (Darmstadt, Germany). Trizma-Base was purchased from Sigma (St. Louis, MO, USA).

Octyl-Sepharose and Phenyl-Sepharose were gifts from Pharmacia Biotech (Uppsala, Sweden). Serum samples mixed from 50 different patients to obtain a serum pool were purchased from the University Hospital (Uppsala). Antisera against human serum proteins were kindly supplied by Prof. C.B. Laurell (Dept. of Clinical Chemistry, Malmö Hospital, Malmö, Sweden).

2.2. Methods

2.2.1. Methods used for salt-dependent adsorption studies

In order to achieve high reproducibility during the chromatography, an optimized program for adsorption, desorption and rinsing of the adsorbent was executed by means of a programmable FPLC® system (Pharmacia Biotech). Serum was diluted with 0.1 *M* Tris buffer, pH 7.5, to attain a protein concentration of 13.6 mg protein per ml in the presence of the salt to be studied. A column (3×1 cm I.D.) packed with the respective adsorbent was equilibrated with the salt dissolved in 0.1 *M* Tris buffer. At a flow-rate of 0.64 ml per min, 1 ml of sample was loaded on the column followed by 15 ml of the salt solution on 0.1 *M* Tris buffer. Ten ml of 0.1 *M* Tris buffer was passed through the column at a flow-rate of 0.64 ml per min. Finally, the gel was rinsed with 0.1 *M* NaOH when mercaptopyridine agarose was used and 20% (v/v) ethanol in deionized water for the other adsorbents. The described procedure was repeated at 75% and 50% of the original salt concentrations.

The adsorption studies have been performed in a system with an excess of adsorption sites to avoid the problem of overloading. The total amount of protein loaded on the column was about 2/3 of maximal dynamic capacities for respective adsorbent which have been determined to 28.4 mg protein per ml of gel for Phenyl-Sepharose and 23.5 and 21.0 mg/ml gel for Octyl-Sepharose and mercaptopyridine-derivatized agarose, respectively [4]. Some experiments were performed on butyl-derivatized agarose with dynamic capacity in the same range.

2.2.2. Immunodiffusion

Serum proteins (according to Table 1) from the desorbed material in the chromatography experiment were immunologically identified by the conventional Ouchterlony technique using monospecific antibodies.

2.2.3. Multivariate analysis

2.2.3.1. General information

Principal component analysis (PCA) was performed using SIRIUS 3.0 (Pattern Recognition Systems, Bergen, Norway). The proteins were considered objects and their interaction with the different media with different buffers were taken as variables. This gives the possibility to compare and correlate other protein characteristics, such as carbohydrate content with the adsorption behavior on the media. Different issues were elucidated by choosing different sets of variables [7].

Table 1
Serum proteins

α-2-Macroglobulin	Haptoglobulin
Oroso-mucoid	Chymotrypsin
IgG	Prealbumin
IgA	Complement factor 4
Albumin	Complement factor 3
Transferrin	IgM
α-1-Antitrypsin	β-Lipoprotein
Ceruloplasmin	α-Lipoprotein

Generally, an adsorption of a protein to a medium was given the value one, while unretarded compounds were given the value zero. Normally, the data were standardized before (PCA) by division with the standard deviation. The choice of significant principal component projections and a qualitative validation of the results were done by study of the ''sum-of-squares for all principal components'' histograms together with the corresponding projections.

2.2.3.2. Questions at issue

(A) The adsorption of serum proteins on mercaptopyridine substituted gel in different concentrations of NaCl, KCl, ammonium acetate and sodium acetate was analyzed by PCA. The same investigation was performed for Phenyl-Sepharose and Octyl-Sepharose.

(B) Adsorption patterns for the different media equilibrated in the same salt.

2.2.3.3. Interpretation of PCA projections

The optimal two-dimensional projection of principal components to best describe certain variables or objects can be easily chosen by studying the sum-of-squares histograms. In the loading plot the projections of the variable vectors from the multivariate space to this two-dimensional space is visualized. The score plot describes the objects in a similar way. For the interpretation of the information in the two-dimensional charts there are some guidelines [7].

1. Variables and object positions should be considered as the points of vectors with start in the origin. The very point is in the center of the name or designation. Thus a variable or an object can be said to point in a direction.
2. Variables pointing in the same direction have a similar variation over the objects. This covariation is a possible correlation but can also be a result of the experimental design or due to a third underlying variable. Variables pointing in the opposite directions have a negative covariation; a high value in one variable corresponds to a low value in the other and vice versa over the objects.
3. Variables with orthogonal directions are independent provided that the scaling of the two axes are the same.
4. Variables and objects can be grouped in classes based on their positions in the plot. Clusters in the plot indicate classes, i.e., similar adsorptions patterns.
5. In this investigation the proteins are considered to objects and the combinations of matrix, buffer salt and salt concentration considered to be variables.

2.2.3.4. Selectivity

The proteins of a defined mixture adsorb or do not adsorb to a substituted medium under certain conditions. This adsorption pattern can be interpreted as describing the selectivity of the medium under the conditions in question. (This definition of selectivity should not be mixed up with that used e.g., in gel filtration). In this investigation the serum proteins work together as a probe and the pattern of adsorbed and nonadsorbed proteins is one description of the selectivity. In hydrophobic interaction chromatography this adsorption pattern can not usually be correlated to a single linear selectivity scale but, by the principal component analysis approach, it is quite possible to compare and classify the selectivities of the different media. Two selectivities can be determined to be similar or different and there is also a possibility to hint whether e.g., a high carbohydrate content in a molecule correlates to the interaction with the medium. By use of the probe a multidimensional selectivity map can be generated. Any new medium under any new condition can thus be placed on the map and compared to other media.

3. Results

3.1. The effect of different type of salts and salt concentrations on the adsorption of serum proteins on respective adsorbent

The protein adsorption pattern on the mercaptopyridine gel when using different buffers and different buffer concentrations is shown in Fig. 1. The position of a certain combination of salt and concentration on the plot corresponds to an adsorption pattern over all the tested proteins. The following conclusions can be drawn: in 1.5–3 *M* potassium chloride and in 1–2 *M* sodium chloride virtually the same proteins adsorb to the column or pass the column unretarded. There is an indication, that a

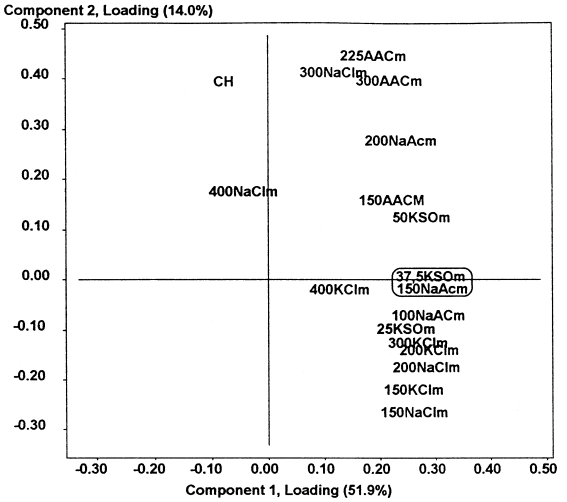

Fig. 1. Loading plot of the adsorption of test proteins to mercapto-pyridine agarose. The designation of the variables is built up of the molarity ×100, the salt and the gel medium. The salts were potassium chloride (KCl), sodium chloride (NaCl), ammonium acetate (AAC), potassium sulphate (KSO) and sodium acetate (NaAc). The gels were mercaptopyridine agarose (m), Phenyl-Sepharose (f), Octyl-Sepharose (o) and butyl agarose (b). Variables very near or covering each other were separated for readability. This is indicated by a surrounding frame. The center of the frame indicates the position of the included variables. The projection shows two main kinds of selectivity, one in moderate and weak sodium and potassium chloride the other in ammonium acetate and 3 *M* sodium chloride. The former is promoted by a low and the latter by a high hydrocarbon content of the test molecules. Quite a special interaction takes place in 4 *M* sodium chloride. The nonlinear change of selectivity with concentration of sodium chloride indicates conformational changes among some of the tested proteins.

lower carbohydrate content of a protein promotes the binding to this gel under these conditions.

There is some change in the adsorption pattern when applying 4 *M* potassium chloride and with 4 *M* sodium chloride a quite unique selectivity pattern is indicated. This is further supported in Fig. 10:1 where the pattern of the protein interactions with the gel is shown to be expressed mainly along principal component three, while the main part of the variations is expressed in the plane spanned by principal component one and two. It is also well demonstrated that 3 *M* sodium chloride and ammonium acetate give a similar selectivity quite distinguished from the previously mentioned. In this environment a high carbohydrate content covariates with the adsorption to the mercapto-pyridine gel.

The adsorption pattern of the tested proteins on Octyl-Sepharose with different salts and salt concentrations is illustrated in Fig. 2. The change of the adsorption pattern on this medium is demonstrated to be related almost linearly to the ionic strength for all salts. The higher efficiency of ammonium acetate to promote binding is clearly shown. The carbohydrate content of a molecule generally promotes binding at higher salt concentrations but varies inversely to the binding at low salt concentrations. This conclusion is made in the following way. In the two-dimensional projection showed in the Figure imagine vectors footed in the origin with points into the different variables, such as ''interaction in a certain buffer'' or ''carbohydrate content'' of the molecules. For instance for the buffer conditions in the upper right corner these vectors are clustered which means that over all tested proteins they all have the same adsorption pattern: virtually the same molecules interact or do not interact with the matrix. The vector

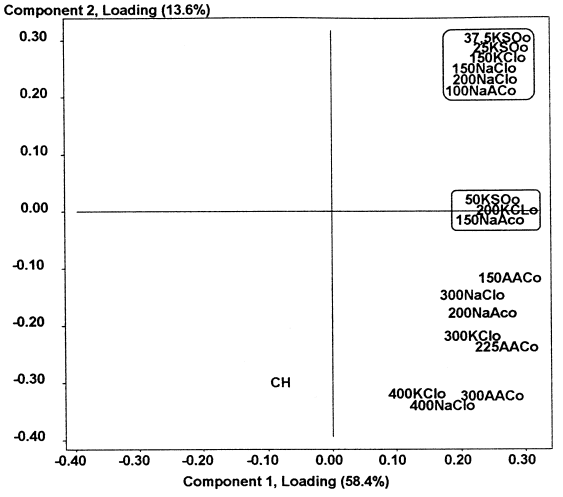

Fig. 2. Adsorption on Octyl-Sepharose. The change of adsorption pattern on this medium is related to the ionic strength in a linear fashion in all salts. The higher efficiency of ammonium acetate to promote binding is clearly demonstrated. Carbohydrate generally promotes binding at higher salt concentrations and prevents binding at low concentrations. Only 3 *M* ammonium acetate is needed to match the interaction of 4 *M* KCl and 4 *M* NaCl. For further reading about the interpretation of PCA-plots refer to Section 3.1 and Ref. [7]. Designations as in Fig. 1.

of the carbohydrate content of the tested substances is pointing in the opposite direction which means that the carbohydrate content varies inversely to the interaction under the mentioned conditions: high carbohydrate content means low affinity and vice versa. On the other hand, in 4 *M* salt the carbohydrate vector points in nearly the same direction as the interaction vectors, leading to the conclusion that high carbohydrate content covariates with this interaction. Moreover only 3 *M* ammonium acetate is needed to match the interaction of 4 *M* potassium chloride and 4 *M* sodium chloride. As on the mercapto-gel, 4 *M* sodium chloride is most effective as an agent to promote adsorption. On Octyl-Sepharose, on the other hand, no peculiar effect of 3 *M* sodium chloride is detected and generally there is a more linear change of the selectivity, seemingly mainly due to the changes in ionic strength, quite in accordance with earlier publications [7] where the adsorption on this gel is shown to be entirely correlated to the content of hydrophobic amino acids of the proteins.

The graph in Fig. 3 illustrates the selectivities on Phenyl-Sepharose. 1.5 *M* sodium acetate and the higher concentrations of chlorides and ammonium acetate give the same selectivity. The selectivities in

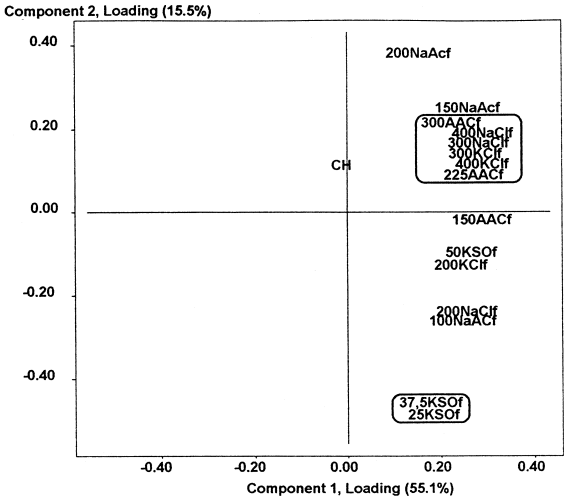

Fig. 3. Adsorption on Phenyl-Sepharose. Weak sulphate buffers give a special selectivity on this matrix. The selectivity is much more sensitive to changes in sodium acetate than ammonium acetate concentration. Designations as in Fig. 1.

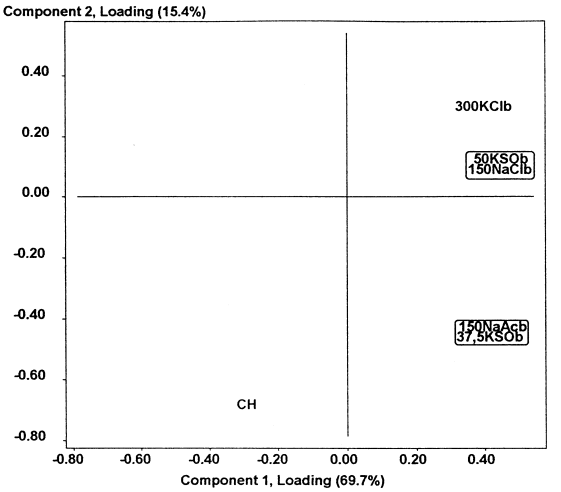

Fig. 4. Adsorption on butyl-agarose. Two different selectivities are demonstrated. Designations as in Fig. 1.

potassium sulphate and sodium acetate is most sensitive to changes in buffer concentration. On this medium there is virtually no correlation between the selectivity and the carbohydrate content of the molecules. This is confirmed in Fig. 10:3 where the variation of the carbohydrate content of the molecules is shown to be almost perpendicular to the main variation, i.e., uncorrelated.

The interaction on butyl-substituted gel (Fig. 4) for some salts reveals still another type of pattern where low carbohydrate content favours binding in high buffer concentrations as opposed to the case of mercapto- and octyl-substituted media. For the significance of the chosen projection see Fig. 10:4.

3.2. The adsorption pattern of serum proteins on different adsorbents in the presence of the same type of salt

The adsorbents can be basically be divided into three different groups with respect to the adsorption pattern of proteins in the presence of KCl (see Fig. 5): (i) Phenyl-Sepharose and Octyl-Sepharose in combination with high KCl concentration (3–4 *M*); (ii) Octyl-Sepharose in combination with moderate salt concentrations (1.5–3 *M*) and mercapto-pyridine-derivatized agarose in high salt concentration; (iii) butyl-agarose and mercapto-pyridine-aga-

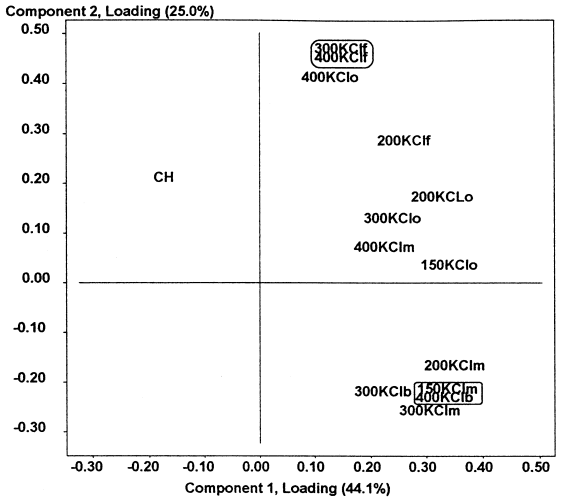

Fig. 5. Adsorption on the different media in different concentrations of KCl. The chart indicates clearly differences in the adsorption mechanisms and the sensitivity to changes in buffer concentration. The selectivities of the butyl and mercaptopyridine agaroses are promoted by a low carbohydrate content and quite separate from those of Octyl and Phenyl Sepharose. Designations as in Fig. 1.

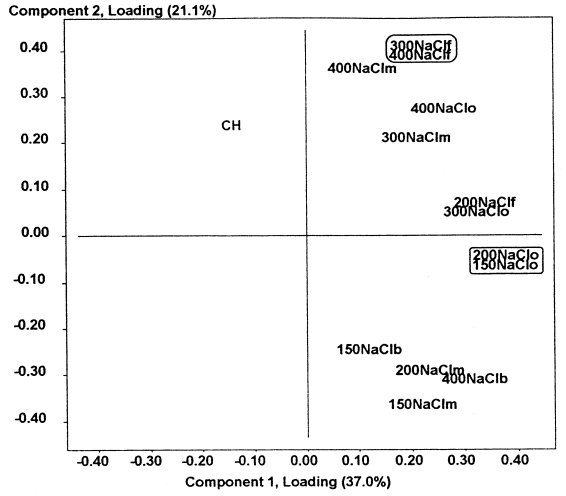

Fig. 6. Adsorption on the different media in different concentrations of NaCl. The selectivities of the butyl and mercaptopyridine gels are similar in low salt concentrations while at higher salt concentrations the selectivity of the maercapto-pyridine matrix tends to be similar to that of the phenyl and octyl media. These shifts of the selectivity with salt concentration can be utilized in the designing of optimal separation in a given application. Designations as in Fig. 1.

rose in combination with 1.5–3 *M* KCl. Low carbohydrate promotes binding in the last group. For the significance of the chosen projection see Fig. 10:5.

In NaCl a similar selectivity is obtained with butyl-agarose in high salt concentration as with mercapto-pyridine-agarose in combination with lower salt concentration (see Fig. 6). Octyl- and Phenyl-Sepharose in combination with 1.5–3 *M* NaCl have the same selectivities pattern while mercaptopyridine-derivatized agarose, Phenyl- and Octyl-Sepharose have the same adsorption pattern at higher salt concentration (3–4 *M*). Fig. 10:6 reveals that for some conditions a substantial fraction of the variation is expressed in other projections, indicating different interaction modes of the chromatographic matrices in sodium chloride.

When ammonium acetate is combined with the different adsorbents (see Fig. 7), the resulting adsorption patterns can be divided into three distinctive categories (butyl-agarose was not analyzed): (i) Phenyl-Sepharose; (ii) Octyl-Sepharose; (iii) mercaptopyridine-derivatized agarose.

Thus in this environment a mainly ligand gener-

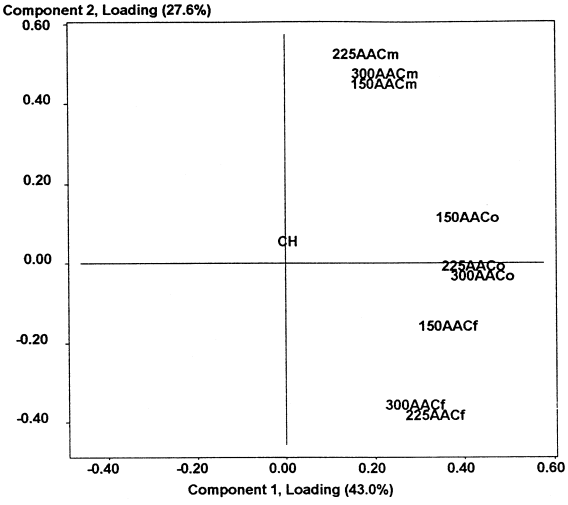

Fig. 7. Adsorption in ammonium acetate on Octyl-Sepaharose, Phenyl-Sepharose and mercapto-pyridine agarose. The selectivities in this environment are very medium-specific and not so sensitive to changes in concentration. The carbohydrate content of the molecules is not a significant factor in the interactions. The separate interaction mechanism of the three tested media is clearly demonstrated. Designations as in Fig. 1.

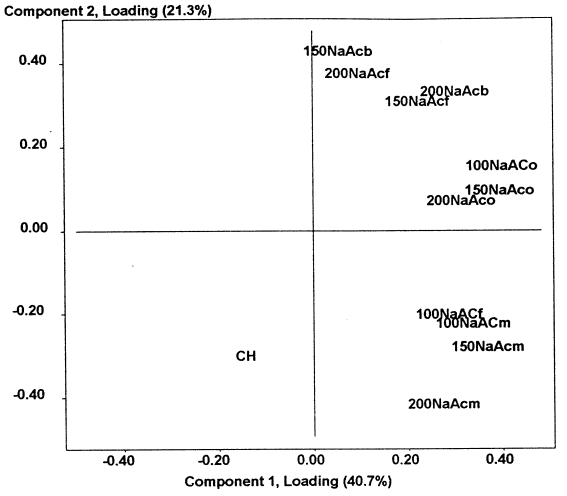

Fig. 8. Changing sodium acetate for ammonium acetate changes the interactions drastically. At low salt concentrations Phenyl-Sepharose acts as the mercapto-pyridine medium. At moderate and high salt concentration the selectivity of Phenyl-Sepharose resembles that of the butyl-agarose.

ated set of selectivities is generated! Moreover, the carbohydrate content of the proteins is quite uncorrelated (Fig. 10:7)

By changing the salt to sodium acetate drastic changes can be observed in the adsorption pattern compared to the other buffers. Butyl-agarose can now be classified together with Phenyl- and Octyl-Sepharose (see Fig. 8). Mercaptopyridine-derivatized agarose at both high and low salt concentrations and Phenyl-Sepharose at low salt concentration show similar adsorption patterns.

3.3. Correlation between carbohydrate content of adsorbed proteins and the adsorption pattern onto mercaptopyridine-derivatized agarose

A high carbohydrate content gives a strong adsorption in the presence of ammonium acetate and 3 *M* NaCl, while a low carbohydrate content will promote the binding in lower potassium and sodium chloride concentrations. The highest concentration of

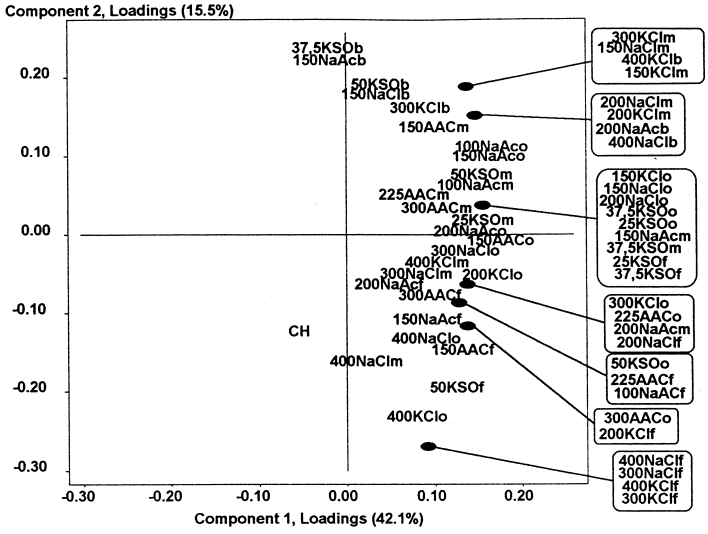

Fig. 9. Principal component map of the investigated separation media in all salts and all concentrations. In one end of the map there is a concentration of butyl-agarose and mercapto-pyridine agarose. Mercapto-pyridine agarose and Phenyl-Sepharose in low salt concentrations are grouped in the middle together with Octyl-Sepharose while Phenyl-Sepharose in high salt is found in the other end of the map. The selectivity span of the media and its variation in the different buffer salts can be easily followed. Designations as in Fig. 1. The frames collecting variables at very similar positions are moved out of the projection for readability and the corresponding positions indicated by a spot.

potassium and sodium chloride and sodium acetate of all investigated concentrations are not especially correlated with adsorption of glycoproteins.

3.4. Classification of the adsorbents based on all parameters investigated

Fig. 9 classifies the adsorbents investigated based on all salts and salt concentrations used. In one end of the selectivity map there is a concentration of butyl-agarose and mercapto-pyridine agarose. Octyl-Sepharose and mercapto-pyridine agarose and Phenyl-Sepharose in low salt concentration are grouped in the middle. Phenyl-Sepharose in high salt is found in the other end of the map.

4. Discussion

Adsorption of proteins onto surfaces has been studied from different aspects to increase the understanding of protein surface interactions [8,9]. When complex biological samples are used the interaction studies will be complicated [10–12] and several investigations like measurements of binding constants and thereby interaction energies are difficult to determine. On the other hand analyses of the type of proteins adsorbed to different types of related surfaces under different conditions provide possibilities to evaluate similarities and dissimilarities in the mechanisms of interactions.

The intention of this study was to analyze the influence of solvent additives and microenvironments on the type of proteins adsorbed to respective adsorbent. By classifying the adsorbents according to their adsorption patterns as a result of these additives, conclusions can be made about similarities in protein surface interactions.

To minimize the risk of competitive adsorption an excess of interaction sites has been used which was controlled by determination of dynamic capacity. Despite that, competitive adsorption cannot be eliminated.

Analyzed proteins are proteins which are reversibly adsorbed to the adsorbents which means that the irreversibly bound proteins are not included in this investigation. From analyses of desorption efficiencies from different adsorbents [4] we know that the desorption efficiencies are relatively low on surfaces like octyl- and phenyl- agarose compared to mercaptopyridine-derivatized agarose and butyl-agarose.

From the results obtained from multivariate analyses it is quite obvious that the adsorbents show different selectivity for the proteins in contact with the investigated surfaces. More surprisingly, the same adsorbent shows different selectivity for adsorbed proteins in the presence of different types of salts. Also the concentration of salt strongly influences the selectivity in protein adsorption and this influence is different and dependent both on the separation medium and the buffer salts. These results are difficult to explain only in terms of existing theories for hydrophobic interactions. According to these theories, the salt induces changes in the chemical potential of the proteins according to Arakawa [13] or the surface tension of the surrounding solution will be changed according to Melander and Horváth [14] by addition of different solvent additives. An alternative explanation is that proteins are constantly fluctuating in there conformation and some of those conformations will be favored in the presence of a certain type of salt and salt concentration with concomitant exposure of interactive sites on the surface of the actual protein. When such exposed sites are complementary to the groups on the derivatized gel surface favorable interactions take place. During other conditions those groups are buried into the protein structure [15].

When all parameters investigated were evaluated by multivariate analysis the investigated adsorbents show different adsorption characteristics. Due to the great number of variables a substantial part of the variation is scattered in principal components of higher order. A very clear impression, though, is that a desired specificity might well be obtained in several different ways by combining the separation media with different buffer salts at certain concentrations. This does not mean, however, that all possible combinations will be feasible for practical reasons such as cost of chemicals, convenience and environmental considerations. It is quite obvious that mercapto-derivatized agarose can be a most valuable complement to the commercially available alternatives in order to provide the degrees of freedom that is needed to get an optimal separation in a certain application.

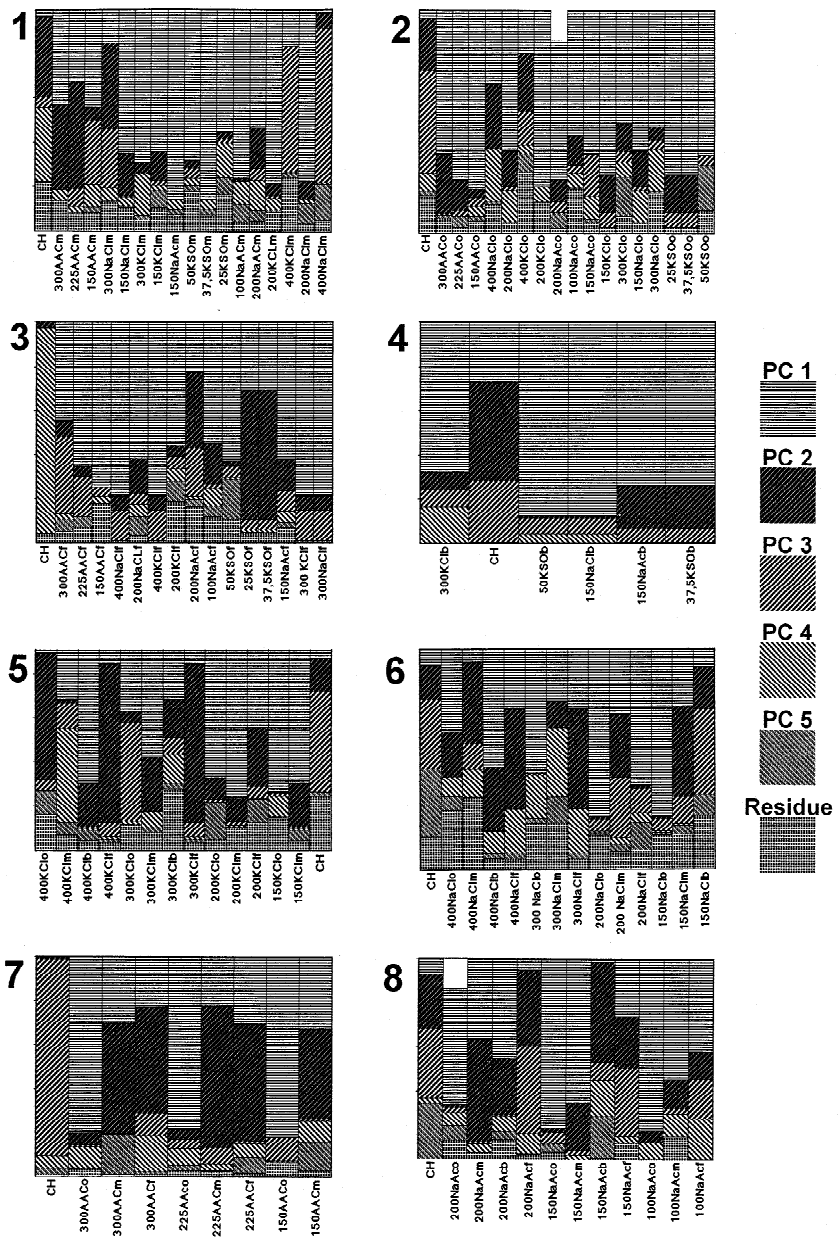

Fig. 10. 1–8 Sum-of-squares histograms of the data of Figs. 1–8, respectively. The diagrams reveal how much of the varation of all the variables in all of the figures that is captured in the different principal components. It is clearly seen that the projections of principal components one and two generally express most of the varation of all varables. Exceptions, where a variation of a variable is expressed mainly along other principal components, show that this variation is poorly correlated to the others. The carbohydrate content of the tested proteins e.g., is not correlated to the chromatographic interaction on the phenyl-gel (Fig. 3 and 10:3) and not in ammonium acetate on any of the tested gels (Fig. 7 and 10:7). It is highly important on the butyl-gel (Fig. 4 and 10:4) and has some influence in the rest of the cases.

The obtained results indicate that all four of the tested gels work according to different mechanisms. These differences are most likely due to the interaction of the combination of matrix, spacer and ligand on one hand with the different features of the proteins such as e.g., conformation, the distribution of hydrophobic amino acids and the content of carbohydrate. By the choice of buffer salts the optimal mechanism for a certain application can be utilized.

4.1. The analysis technique

The approach to utilize principal component analysis in the way presented here may be conceived as unconventional and needs further discussion.

To choose the different proteins as objects and the result of their interaction with the investigated chromatographic matrices in the different environments as variables, opens up several analytical possibilities. In a conceptual way this choice means that for a protein an interaction with a gel could be seen as an attribute of the protein just like e.g., molecular mass, isoelectric point or carbohydrate content. And this gives immediately the possibility, by the principal component analysis, to discover possible correlations between the attributes. On the other hand, the technique also permits a classification of the involved proteins by the adsorption pattern formed by all tested conditions. One crucial point in principal component analysis is to find the projection which tells most about the issue. By definition, a projection formed by the first and the second principal components always reveals most information about the entire data set which is why we choose this projection generally in this investigation. This decision was supported when we used the graphic, analogous information of the accumulated sum-of-squares plots to judge the significance of the different possible projections for the study of a certain set of variables. The decision between significance or nonsignificance is never black-or-white and the final judgement has always to be done by the interpreter. We think that the graphic information is clearer and at a glance gives a better picture than tables of eigenvectors, eigenvalues and other digital information, to judge the possible significance of the different PCA-plots for any variable and, inversely, the possible significance of a variable in a plot. In Fig. 10 the sum-of-squares plots corresponding to Figs. 1–8 are shown. From this Figure it is obvious that a projection of the first two principal components in all cases describes the main part of the variation over the tested proteins under the different conditions. However it is also revealed that in strong sodium chloride another mechanism is working, which would be better studied by plotting principal components one against three (Figs. 1 and 10:1). This conclusion can be drawn because the variation of the adsorption in this environment is extended in another direction in the space which corresponds to another adsorption pattern over the proteins, thus being the result of another type of interaction. Another example is the carbohydrate content of the tested proteins which is found to be not correlated to the chromatographic interaction on the phenyl-gel (Figs. 3 and 10:3) and not in ammonium acetate on any of the tested gels (Figs. 7 and 10:7). Carbohydrate is highly important for the interaction on the butyl-gel (Figs. 4 and 10:4) and has some influence in the rest of the cases.

5. Conclusions

In the process of developing new adsorbents for chromatography several concepts are possible. Before starting with time-consuming experiments with the intention of finding more or less specific interactions between proteins and adsorbents, we have chosen to investigate the adsorbents with respect to their adsorption characteristics by use of more complex biological samples containing representative proteins from physicochemical point. By use of multivariate analyses the complex evaluation of results and classification of the adsorbents were made. Strong evidence was obtained for different mechanisms operating on the adsorbents and under different buffer conditions.

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